

INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

Final Guideline Release: This guideline is available from the U.S. Government Printing Office, Washington, DC 20402 on disks or paper copies: call (202) 512-0132. This guideline is also available electronically in PDF (portable document format) from EPA's Internet Web site at <http://www.epa.gov/opptsfrs/home/guidelin.htm>.

OPPTS 870.2600 Skin sensitization.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are OPPTS Harmonized Test Guidelines Series 870, Guideline 870.2600 Skin Sensitization, dated August 1998; 40 CFR 798.4100 Dermal Sensitization; OECD 406 Skin Sensitization (adopted July 1992); and OECD 429 Skin Sensitization: Local Lymph Node Assay (adopted April 2002).

(b) **Purpose.** The purpose of the selected test is to identify substances with skin sensitization potential. Determination of the potential to cause or elicit skin sensitization reactions (allergic contact dermatitis) is an important element in evaluating a substance's toxicity. Information derived from skin sensitization tests serves to identify possible hazards to a population exposed repeatedly to a test substance. Testing is not required if the test material is a known skin sensitizer. If it is suspected that the test material is a strong dermal irritant, see OPPTS 870.1000, paragraph (d)(2)(iii).

(c) **Definitions.** The following definitions apply to this test guideline. The definitions in Section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) also apply to this test guideline.

Challenge exposure is an exposure of a previously treated subject to a test substance following an induction period to elicit a contact hypersensitivity response.

Induction exposure is the administration of a test substance to the test subject with the intention of inducing contact sensitization.

Induction period is a period of at least 1 week following an induction exposure during which sensitization may develop.

Skin sensitization (allergic contact dermatitis) is an immunologically mediated cutaneous reaction to a substance. In the human, the responses may be characterized by pruritis, erythema, edema, papules, vesicles, bullae, or a combination of these. In other mammalian species, the reactions may differ and only erythema and edema may be seen.

Stimulation index (SI) is the ratio of ³H-methyl thymidine or ¹²⁵I-iododeoxyuridine (¹²⁵IU) incorporation into test group lymph nodes relative to that recorded for solvent/vehicle control group lymph nodes.

(d) **Test procedures**—(1) **Methods.** Any of the following test methods is considered to be acceptable:

- (i) Local Lymph Node Assay (LLNA) test, or
- (ii) Guinea-Pig Maximization Test (GPMT), or
- (iii) Buehler test.

(2) **Choice of assays.** See OPPTS 870.1000 for a general discussion of factors to be considered prior to performing the test. In addition, the following considerations apply:

(i) The LLNA (see references in paragraphs (g)(1) through (g)(6) of this guideline) is a preferred alternative method, where applicable, to the traditional guinea pig test because it demonstrates an equivalent prediction of human allergic contact dermatitis as compared to the other sensitization tests, provides quantitative data and an assessment of dose-response, gives consideration to animal welfare concerns, and is suitable for testing colored substances. It should be recognized that there are certain testing situations that may necessitate the use of traditional guinea pig tests. The tester should note that the LLNA may not be appropriate for all types of test materials, such as certain metallic compounds, high molecular weight proteins, strong dermal irritants and materials that do not sufficiently adhere to the ear for an acceptable period of time during treatment. When using the LLNA, particular care should be taken to ensure that hydrophilic materials are incorporated into a vehicle system that wets the skin and does not immediately run off. Thus, wholly aqueous vehicles or test materials and runny liquids are to be avoided. In all instances, the tester must document that appropriate techniques were used to facilitate adherence to the mouse ear for an adequate exposure duration. It may be possible to use the LLNA to test some of these materials if appropriate techniques are used to facilitate adherence.

(ii) In situations for test materials where the LLNA is not applicable or may provide unreliable or problematic results, the GPMT or Buehler tests are recommended (see references in paragraphs (g)(7) through (g)(14) of this guideline).

(iii) Although the LLNA, GPMT, or Buehler tests are considered to be acceptable tests, it is recognized that other tests may give useful results. If other tests are used, the investigator must provide justification/reasoning for use of other procedures and methods and protocols must be provided. A positive and negative control group must be included in each test.

(e) **Test methods—(1) LLNA method—(i) Principle of the method.** The basic principle underlying the LLNA is that skin sensitizers induce proliferation of lymphocytes in the lymph nodes draining the site of chemical application. Generally, under appropriate test conditions, this proliferation is proportional to the dose applied, and provides a means of obtaining an objective, quantitative measurement of sensitization. The test measures cellular proliferation as a function of *in vivo* radioisotope incorporation

into the DNA of dividing lymphocytes. The LLNA assesses this proliferation in the draining auricular lymph nodes located in the cervical region at the bifurcation of the jugular vein. Lymphocyte proliferation in test groups is compared to that in concurrent solvent/vehicle-treated controls. A positive control is added to each assay to provide an indication of appropriate assay performance.

(ii) **Animal selection**—(A) **Sex and strain of animals.** Young adult female mice (nulliparous and non-pregnant) of the CBA/Ca or CBA/J strain should be used at age 8–12 weeks. All animals are to be age-matched (preferably within a one-week time frame). Females are used because the existing database is predominantly based on this gender. Males and other strains of mice should not be used until it is sufficiently demonstrated that significant strain-specific and/or gender-specific differences in the LLNA response do not exist.

(B) **Housing and feeding.** The temperature of the experimental animal room should be 21 ± 3 °C and the relative humidity 30–70%. When artificial lighting is used, the light cycle should be 12 hours light: 12 hours dark. For feeding, standard laboratory mouse diets are to be used with an unlimited supply of drinking water. The mice must be acclimatized for at least 5 days prior to the start of the test. Animals must be housed individually. Healthy animals are randomly assigned to control and treatment groups having statistically homogeneous body weights. The animals are uniquely identified prior to being placed on study. Although a variety of techniques exist to uniquely mark mice, any method that involves identification via ear marking (e.g., ear tags) must not be used.

(iii) **Test conditions**—(A) **Preparation of doses.** Solid test substances are to be dissolved in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the animals. Stable suspensions might also be acceptable. Liquid test substances may be dosed directly or diluted prior to dosing. Fresh preparations of the test substance are to be prepared daily unless stability data demonstrate the acceptability of storage.

(B) **Solvent/vehicle.** The solvent/vehicle is to be selected on the basis of maximizing the test concentration while producing a solution/suspension suitable for application of the test substance. In order of preference, recommended solvents/vehicles are acetone/olive oil (4:1 v/v), *N,N*-dimethylformamide, methyl ethyl ketone, propylene glycol, and dimethyl sulfoxide, but others may be used if appropriately justified. The selected solvent/vehicle must not interfere with or bias the test result and should be selected to achieve the maximum concentration/skin exposure of the test substance. Ensure that hydrophilic materials are incorporated into a vehicle system that wets the skin and does not immediately run off. Thus, wholly aqueous vehicles are to be avoided.

(C) **Controls.** (1) Concurrent negative (solvent/vehicle) and positive controls are to be included in each test. In some circumstances, it may be useful to include a naive control. Except for treatment with the test substance, animals in the control groups are to be handled in an identical manner to animals of the treatment groups.

(2) Positive controls are used to ensure the appropriate performance of the assay. The positive control must produce a positive LLNA response at an exposure level expected to give an increase in the stimulation index (SI) of three or greater ($SI \geq 3$) over the solvent or vehicle control group. The positive control dose is to be chosen such that the induction is clear but not excessive. Preferred positive control substances are hexyl cinnamic aldehyde (HCA) and mercaptobenzothiazole. There may be circumstances where, given adequate justification, other positive control substances may be used. However, benzocaine should not be used as a positive control in the LLNA.

(3) The positive control substance is tested in the vehicle that is known to elicit a consistent response (i.e., acetone/olive oil). If a non-standard vehicle (chemically relevant formulation) is used with a positive control, the non-standard vehicle (chemically relevant formulation) must be tested for a local lymph node response prior to the initiation of the study and the results reported.

(iv) **LLNA test procedure—(A) A minimum of five animals are used per dose group.** At least three consecutive doses of the test substance are to be used. A solvent/vehicle control group and a positive control group are also required. Doses are normally selected from within the concentration series 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, 0.1%. In general, dose selection is based on factors such as toxicity, solubility, irritancy and any other available information such as the results of other testing and structure-activity relationships. To avoid false negatives, test as high a concentration as possible. Generally, the maximum concentration tested is the highest achievable level that avoids overt systemic toxicity and excessive local irritation. To identify the appropriate maximum test substance dose, an initial toxicity test, conducted under identical experimental conditions except for an assessment of lymph node proliferative activity, may be necessary. To support an ability to identify a dose-response relationship, data must be collected on at least three test substance treatment doses, in addition to the concurrent solvent/vehicle control group. Where the LLNA study results are negative, the concurrent positive control must induce a $SI \geq 3$ relative to its solvent/vehicle-treated control.

(B) **LLNA experimental procedure.** The LLNA experimental procedure is to be performed by appropriately trained staff as follows:

(1) Day 1. Record the body weight of each mouse prior to dermal applications. Apply 25 μ L/ear of the appropriate dilution of the test sub-

stance, or the positive control, or the solvent/vehicle control alone to the dorsum of both ears. A positive displacement pipettor may facilitate application of the test material.

(2) Days 2 and 3. Repeat the application procedure as carried out on day 1.

(3) Days 4 and 5. No treatment.

(4) Day 6. Record the body weight of each mouse. Inject 250 μ L of sterile phosphate buffered saline (PBS) containing 20 μ Ci of 3 H-methyl thymidine or 250 μ L PBS containing 2 μ Ci 125 IU and 10^{-5} M fluorodeoxyuridine into each experimental mouse via the tail vein. Five hours later, the draining (auricular) lymph node of each ear is excised and pooled in PBS for each animal. A single cell suspension of lymph node cells (LNC) is prepared for each mouse. The single cell suspension is prepared in PBS by either gentle mechanical separation through 200-mesh stainless steel gauze or another acceptable technique for generating a single cell suspension. The LNC are washed twice with an excess of PBS and the DNA precipitated with 5% trichloroacetic acid (TCA) at 4 $^{\circ}$ C for approximately 18h.

(5) For the 3 H-methyl thymidine method, pellets are resuspended in 1 mL TCA and transferred to 10 mL of scintillation fluid. Incorporation of 3 H-methyl thymidine is measured by B-scintillation counting as disintegrations per minute (dpm) for each mouse and expressed as dpm/mouse. For the 125 IU method, the 1 mL TCA pellet is transferred directly into gamma counting tubes. Incorporation of 125 IU is determined by gamma counting and also expressed as dpm/mouse.

(C) **Observations.** At a minimum, observe mice once daily for any clinical signs, either of local irritation at the application site or of systemic toxicity. Weighing mice prior to treatment and at the time of necropsy will aid in assessing systemic toxicity. All observations are systematically recorded, with records being maintained for each individual mouse.

(D) **Measurements and calculation of results.** (1) The proliferative response of lymph node cells from the pooled lymph nodes of each individual animal is expressed as the number of radioactive disintegrations per minute (dpm) per animal, subtracting out any background dpm. Then the group mean dpm, along with an appropriate measure of inter-animal variability (i.e., mean \pm standard deviation), is calculated for each test group (i.e., positive, solvent/vehicle, and any other control groups) and the solvent/vehicle group. Final results are expressed as the SI which is calculated as a ratio (i.e., SI = mean dpm of test group divided by mean dpm of solvent/vehicle control group).

(2) In addition to an assessment of the magnitude of the ratio estimate, SI, conduct statistical analyses which include both an overall assess-

ment (e.g. ANOVA) of the dose-response relationships and pairwise comparisons of the SIs of the test groups, positive control group and any other control group versus that of the solvent/vehicle control group. In choosing an appropriate method of statistical analysis, the investigator should be aware of possible inequality of variances and other related problems that may necessitate a data transformation or a nonparametric statistical analysis.

(v) Data interpretation and reporting for LLNA—(A) Data Interpretation. (1) A substance is regarded as a skin sensitizer in the LLNA if at least one concentration of the test material results in a 3-fold or greater increase in ^3H -methyl thymidine or ^{125}IU incorporation in the lymph node cells of test group lymph nodes relative to that recorded for solvent/vehicle control lymph nodes, as indicated by the SI. However, the magnitude of the SI should not be the sole factor used in determining the biological significance of a skin sensitization response. A quantitative assessment must be performed by statistical analysis of individual animal data in order to provide a more complete evaluation of the test substance (see paragraph (e)(1)(iv)(D)(2) of this guideline). Factors to be considered in evaluating the biological significance of a response or outcome of the test include the results of the SI determinations, statistical analyses, the strength of the dose-response relationship, chemical toxicity, solubility, and the consistency of the solvent/vehicle and positive control responses.

(2) Strong irritants may yield false positive results in the LLNA due to the initiation of a significant lymphocyte proliferation. However, the dose-response information from the assay may help to uncover a strong irritant response since, for instance, it has been shown that the proliferation induced by irritation usually results in a shallow dose-response relationship. Concurrent evaluation of ear swelling may also provide helpful information on differentiating weak sensitizers from strong irritants.

(B) Test report. The test report for LLNA must contain the following specific information:

(1) Test substance. (i) Identification data and CAS number, if known, and EPA registration number, if applicable;

(ii) Physical nature and purity;

(iii) Physicochemical properties relevant to the conduct of the study;

(iv) Stability of the test substance, if known; and

(v) Lot number of the test substance.

(2) Solvent/vehicle. (i) Solvent/vehicle used and its purity;

(ii) Justification for choice of solvent/vehicle, if appropriate; and

(iii) Solubility and stability of the test substance in the solvent/vehicle.

(3) Test animals. (i) Strain of mice used;

(ii) Acclimation information;

(iii) Number, age, and sex of mice;

(iv) Source, housing conditions, diet, etc.;

(v) Individual body weight of the animals at the start and end of the test, including body weight range, mean, and associated error term for each group;

(vi) Health and microbiological/pathogen status of the mouse; and

(vii) Details of animal food and water quality;

(4) Test conditions. (i) Details of test substance preparation;

(ii) Details of the administration of the test substance;

(iii) Detailed description of treatment and sampling schedules; and

(iv) Methods for measurement of toxicity.

(5) Results. (i) Positive and negative (solvent/vehicle) control data in tabular form;

(ii) Data from range-finding study, if conducted;

(iii) Doses used;

(iv) Rationale for dose level selection;

(v) Signs of toxicity;

(vi) Dpm/mouse values for each mouse within each treatment group and control group;

(vii) Group mean dpm/mouse and associated error term for each treatment group and control group;

(viii) The SI calculated, compared to the concurrent solvent/vehicle control group, for each test substance treatment dose group, the concurrent positive control group, and any other concurrent control group;

(ix) Individual mouse dpm data must be presented in tabular form, along with the group mean dpm, its associated error term and the SI for each dose group;

(x) Criteria for considering studies as positive or negative (including information on any qualitative or quantitative measure of ear swelling);

